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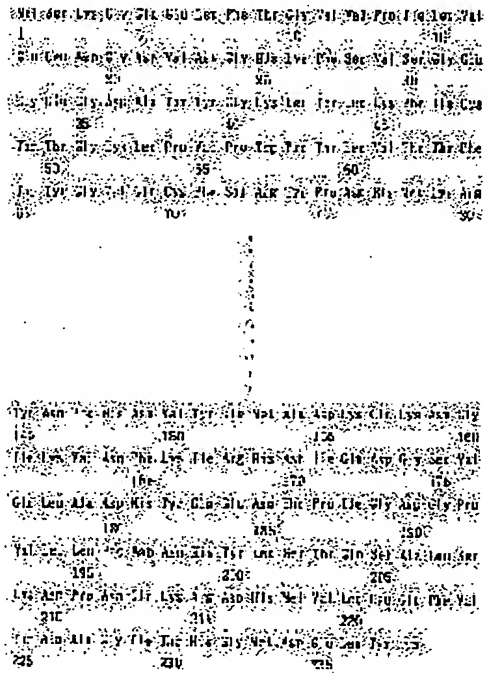
(54) FLUORESCENT PROTEIN

(57)Abstract:

PROBLEM TO BE SOLVED: To obtain a fluorescent protein capable of being expressed even by the culture of a host cell at a high temperature (37°C), emitting stronger fluorescent light than those of conventional fluorescent proteins (GFP), and useful as a labeling agent for the analyses of protein localization in live cells, a reporter for the analyses of promoters, etc., by introducing two mutation amino acids into a wild type GFP.

SOLUTION: This fluorescent protein is obtained by mutating the No. 147 serine and the No. 65 serine of the cDNA of a wild type GFP with proline and threonine, respectively, by a site-specific mutation method, etc., transforming Escherichia coil with a plasmid containing the obtained GFPcDNA and subsequently expressing the mutated GFP containing an amino acid sequence of the formula in the Escherichia coil at a high temperature (37° C). The fluorescent protein emits about three-fold fluorescent light that of S65T mutant,



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is contained in a higher concentration than that of the S65T mutant, when expressed in the cell, and emits the fluorescent light under a high temperature (37°C).

LEGAL STATUS

[Date of request for examination]

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[Date of final disposal for application]

[Patent number]

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TECHNICAL FIELD

[The technical field to which invention belongs] This invention relates to the alteration fluorescence protein with which fluorescence intensity was raised, its activity, etc.

[Translation done.]

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MEANS

[Means for Solving the Problem] In order to solve the above-mentioned technical problem, this invention persons introduced variation into GFPcDNA (array number: 1) of a wild type at random, and performed isolation of cDNA which is carrying out the code of the GFP which emits strong fluorescence out of it. That is, this invention persons prepared GFPcDNA which introduced variation at random by the PCR method, included this in a plasmid, and introduced into Escherichia coli. Subsequently, a clone which emits bright fluorescence especially at 37 degrees C by discovered GFP was selected, plasmids introduced from this clone were collected, and a base sequence of the variation GFP included in a plasmid was analyzed. Consequently, it found out that this variation existed in a location where a serine which is the 147th amino acid of a wild type GFP is replaced by proline, and separated from a chromophore array of GFP in the variation GFP from which this invention persons were obtained.
[0008] Subsequently, in addition to variation of this 147th amino acid, this invention persons built GFPcDNA (array number: 3) which introduced variation (variation to a threonine of the 65th serine) introduced into "S65T variant" which emits strong fluorescence, in order to isolate GFP which emits still stronger fluorescence. and introduced into Escherichia coli a plasmid containing GFPcDNA which has these two variation, this variation GFP (an amino acid sequence -- array number: -- shown in 2) was made to discover within Escherichia coli under an elevated temperature (37 degrees C), and measurement of that fluorescence intensity and concentration was performed. Consequently, intracellular abundance was also found out this invention persons increasing notably as compared with "S65T" variant which the amount of luminescence of this variation GFP used as contrast, and increasing notably. That is, this invention persons succeeded in manufacturing protein which can become a fluorescence mold and emits very strong fluorescence rather than the known variation GFP under an elevated temperature (37 degrees C) by introducing two variation into GFP of a wild type.
[0009] Furthermore, this invention persons examined effectiveness in an animal cell of fluorescence protein which has these two variation. That is, cDNA of fluorescence protein with which two variation was introduced into an animal cell expression vector was incorporated, and this was introduced into a mouse origin cell. Subsequently, a fluorescence image of this fluorescence protein that discovered this cell by intracellular after culture under an elevated temperature (37 degrees C) was detected. Consequently, this invention persons found out that this fluorescence protein was more notably [than "S65T variant" which emitted bright fluorescence and this fluorescence moreover used as contrast also within an animal cell] strong.
[0010] Namely, this invention serves as a fluorescence mold under an elevated temperature (37 degrees C), and can emit strong fluorescence. Also in an animal cell, it is related with applicable fluorescence protein and its directions. And more specifically (1) Fluorescence protein which includes an amino acid sequence of a publication in the array number 2, (2) In an amino acid sequence given in the array number 2 1 or some amino acid Deletion, Fluorescence protein including an amino acid sequence (however, the 65th place is a threonine and the 147th place is a proline) replaced or added, (3) DNA which carries out the code of the fluorescence protein of a publication to (1) or (2), Vector which contains DNA of a publication in (4) and (3) (5) It is characterized by having arranged DNA given in (3) on a **-ed promotor's lower stream of a river. (4) Vector of a publication (6) A host cell which holds a vector of a publication to (4), (7) Include

a process which cultivates a host cell of a publication to (6) and collects produced protein. (1) Or a manufacture method of fluorescence protein given in (2) (8) A vector given in (5) is introduced into a host cell. A measuring method of the activity of a **-ed promotor including a process in which fluorescence emitted from this cell is detected, (9) (1) characterized by uniting with a **-ed amino acid sequence, or fluorescence protein given in (2), (10) Fluorescence protein given in (9) is introduced into a cell, and it is characterized by observing distribution in this intracellular one of this fluorescence protein. How to detect targetting activity in intracellular [of a **-ed amino acid sequence], (11) A vector in which DNA which carries out the code of the fluorescence protein of a publication to (9) was inserted possible [a manifestation] is introduced into a host cell. It is related with a method of detecting targetting activity in intracellular [of a **-ed amino acid sequence] characterized by observing distribution in this intracellular one of this fluorescence protein.

[0011]

[Embodiment of the Invention] This invention relates to fluorescence protein including the amino acid sequence by which the 65th serine was replaced by the threonine and the 147th serine was replaced by the proline among the amino acid in the amino acid sequence of a wild type GFP. The protein of this invention emits about 3 times as much fluorescence as "S65T variant" widely put in practical use by having these two variation now. It has the feature of existing by high concentration from "S65T variant" when it is made discovered by intracellular (presumed since not only fluorescence intensity but proteinic stability increased by having two variation). Furthermore, it has the feature that it can become a fluorescence mold under an elevated temperature (37 degrees C).

[0012] Therefore, in the protein of this invention, the 65th place of an amino acid sequence is a threonine, it is important that the 147th place is a proline, and the same protein is also contained in the range of this invention as intrinsically as the 65th place and the protein of this invention suitably obtained substitute, deletion, and by adding in the amino acid of an except the 147th place. As for "being the same protein intrinsically", the amino acid which does not influence fluorescence substantially points out substitute, deletion, or the added protein among the amino acid in the amino acid sequence of the protein of this invention here.

[0013] In addition, it is possible to build DNA which carries out the code of such alteration protein easily using the site-directed-mutagenesis method which is common knowledge technology (experimental-medicine separate volume gene engineering handbook 246-258 (1991)), if it is this contractor, and to obtain the protein of this invention.

[0014] The protein of this invention can be manufactured by the following methods. First, GFPcDNA which replaced the base sequence corresponding to the 65th serine and the 147th serine by the base sequence corresponding to a threonine and a proline among the base sequences (array number: 1) of a wild type GFPcDNA, respectively is produced. Production of this GFPcDNA can be carried out, when the 193rd "the T" of a wild type GFP ("A" of a translation initiation codon "ATG" is set to 1) is replaced by "A" and it replaces the 439th "the T" by "C" by the site-directed-mutagenesis introducing method etc. Subsequently, produced cDNA is included in a suitable expression vector, it introduces into a host cell, and the protein of this invention is made to discover by host intracellular.

[0015] As an expression vector, although there is especially no limit, it sets to Escherichia coli. For example, "pQE30", "pQE31", "pQE32" (QIAGEN K.K. make), Vectors, such as "pET3a", "pET3b", "pET3c" (novagen company make, TAKARA SHUZO), "pGEX-5X-1", "pGEX-5X-2", "pGEX-5X-3" (Pharmacia manufacture), and "pUC118" (TAKARA SHUZO), are used suitably.

[0016] Installation to the host cell of a vector can be performed by methods, such as a conventional method, for example, a calcium phosphate method, the electroporation method, etc.

[0017] Moreover, a vector is introduced, there is especially no limit as a host cell for making the protein of this invention discover, and "HB [101]", "DH5", "TG1", "JM109", "XL1-blue", "BL21 (DE3)", "BL21(DE3) pLysS", etc. can be used in Escherichia coli, for example.

[0018] The protein of this invention made to discover by host intracellular is refined by the following methods, and can be collected. That is, if the protein of this invention exists as fusibility

protein by host intracellular, after destroying a host cell by ultrasonic crushing etc., it is possible to refine by methods, such as ammonium sulfate precipitation and a DEAE cellulose column chromatography. Moreover, if it exists as insoluble protein, it is possible to refine [by dialysis] salt concentration for example, using the same method as the above about the protein by which lowering solubilization is carried out gradually, after solubilizing using modifiers, such as a guanidine hydrochloric acid. In addition, when the protein of this invention is fusion protein with "His-tag" (QIAGEN K.K. make), it is possible to refine with the affinity column chromatography which used "nickel-NTA resin" (QIAGEN K.K. make). Moreover, when it is fusion protein with a glutathione S-transferase (GST), it is possible to perform purification using the affinity which used the glutathione sepharose column.

[0019] Especially the protein of this invention has the high utility value as an indicator. That is, if the protein of this invention is refined as fusion protein with a **-ed amino acid sequence, it introduces into intracellular by technique, such as a microinjection method, and distribution of this fusion protein is observed with time, it is possible to detect the targetting activity in intracellular [of a **-ed amino acid sequence]. As a **-ed amino acid sequence, although there is especially no limit, a targeting signal (for example, a nuclear shift signal, a mitochondrion pre array) etc. is suitable, for example. In addition, besides introducing into intracellular by a microinjection method etc., it is made discovered by intracellular and the protein of this invention can also be used. In this case, the vector in which DNA which carries out the code of the protein of this invention was inserted possible [a manifestation] is introduced into a host cell.

[0020] Moreover, the protein of this invention can also be used for measurement of promotor activity as reporter protein. That is, it is possible to measure a **-ed promotor's activity by building the vector by which DNA which carries out the code of the protein of this invention to a **-ed promotor's lower stream of a river has been arranged, introducing this into a host cell, and detecting the fluorescence of the protein of this invention emitted from this cell. As a **-ed promotor, if it functions by host intracellular, there will be especially no limit.

[0021] As a vector used in detection of the targeting activity of the above-mentioned **-ed amino acid sequence, or measurement of promotor activity Although there is especially no limit, for example by the vector for animal cells "pNEO" (P.Southern, and P.Berg (1982) J.MOl.Appl.Genet.1:327), "pCAGGS" (H. Niwa, KYamamura, and and J.Miyazaki.Gene 108,193-200 (1991)), "pRc/CMV" (in vitro gene company make), "pCDM8" (in vitro gene company make), etc. by the vector for yeast "pRS303", "pRS304", "pRS305", "pRS306", "pRS313", "pRS314", "pRS315", [pRS316] (R.) [S.Sikorski and P.Hieter (1989)] Genetics 122: 19-27, "pRS423", "pRS424", "pRS425", "pRS426" (T.) [W.Christianson, R.S.Sikorski, M.Dante, J.H.Shero,] [and P.Hieter] (1992) Gene 110: 119-122 etc. is used suitably.

[0022] Moreover, there is especially no limit also in the cell which can be used. Yeast cells, Escherichia coli (E.coli) cells, etc., such as various animal cells, for example, an L cell, a BalbC-3T3 cell, a NIH 3T3 cell, a CHO (Chinese hamster ovary) cell, a HeLa cell, a NRK (normal rat kidney) cell, and "Saccharomyces cerevisiae", are used suitably.

[0023] Installation to the host cell of a vector can be performed with conventional methods, such as for example, a calcium phosphate method and the electroporation method.

[0024] The fluorescence of the protein of this invention can be detected with a viable cell. It is possible to perform this detection using a fluorescence microscope (curl TSUAISU AKISHIO photograph filter set 09), image-analysis equipment (ATTO digital image analyzer), etc.

[0025] In addition, DNA which carries out the code of the protein of this invention can also be used for the analysis of the molecule-molecule interaction using the analysis and the energy transfer of folding (folding) of protein.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[The technical field to which invention belongs] This invention relates to the alteration fluorescence protein with which fluorescence intensity was raised, its activity, etc.
[0002]
[Description of the Prior Art] GFP (green fluorescent protein) of the jellyfish (Aequorea victoria) origin is protein which emits strong fluorescence, and since it is possible to use as an indicator of the analysis of the protein localization in a viable cell especially, or to use as a reporter for promotor analysis, it attracts attention dramatically in recent years.
[0003] However, many of discovered GFP(s) did not get used to a fluorescence mold, but the fluorescence intensity of a wild type GFP had the trouble of emitting only feeble fluorescence by intracellular for this reason, when it was greatly dependent on the cell culture conditions at the time of a manifestation, for example, culture temperature was raised to about 37 degrees C in a mammals cultured cell or yeast.
[0004] By the way, it is known that the fluorescence of GFP of a wild type originates in the chromophore (chromophore) which amino acid 3 residue "Ser(65)-Tyr-Gly" followed on a primary array forms.
[0005] for this reason, the 65th serine under this chromophore array is mutated to a threonine, and "S65T variant" changed so that strong fluorescence might be generated from a wild type GFP develops -- having (R. Heim, A.B.Cubitt, and R.Y.Tsien.Nature., 373 663-664 (1995)) -- current -- it is put in practical use widely.
[0006]
[Problem(s) to be Solved by the Invention] This invention makes it a technical problem to offer the fluorescence protein which emits strong fluorescence rather than conventional fluorescence protein under an elevated temperature.
[0007]
[Means for Solving the Problem] In order to solve the above-mentioned technical problem, this invention persons introduced variation into GFPcDNA (array number: 1) of a wild type at random, and performed isolation of cDNA which is carrying out the code of the GFP which emits strong fluorescence out of it. That is, this invention persons prepared GFPcDNA which introduced variation at random by the PCR method, included this in a plasmid, and introduced into Escherichia coli. Subsequently, a clone which emits bright fluorescence especially at 37 degrees C by discovered GFP was selected, plasmids introduced from this clone were collected, and a base sequence of the variation GFP included in a plasmid was analyzed. Consequently, it found out that this variation existed in a location where a serine which is the 147th amino acid of a wild type GFP is replaced by proline, and separated from a chromophore array of GFP in the variation GFP from which this invention persons were obtained.
[0008] Subsequently, in addition to variation of this 147th amino acid, this invention persons built GFPcDNA (array number: 3) which introduced variation (variation to a threonine of the 65th serine) introduced into "S65T variant" which emits strong fluorescence, in order to isolate GFP which emits still stronger fluorescence. and introduced into Escherichia coli a plasmid containing GFPcDNA which has these two variation, this variation GFP (an amino acid sequence -- array

number: -- shown in 2) was made to discover within Escherichia coli under an elevated temperature (37 degrees C), and measurement of that fluorescence intensity and concentration was performed. Consequently, intracellular abundance was also found out this invention persons increasing notably as compared with "S65T" variant which the amount of luminescence of this variation GFP used as contrast, and increasing notably. That is, this invention persons succeeded in manufacturing protein which can become a fluorescence mold and emits very strong fluorescence rather than the known variation GFP under an elevated temperature (37 degrees C) by introducing two variation into GFP of a wild type.

[0009] Furthermore, this invention persons examined effectiveness in an animal cell of fluorescence protein which has these two variation. That is, cDNA of fluorescence protein with which two variation was introduced into an animal cell expression vector was incorporated, and this was introduced into a mouse origin cell. Subsequently, a fluorescence image of this fluorescence protein that discovered this cell by intracellular after culture under an elevated temperature (37 degrees C) was detected. Consequently, this invention persons found out that this fluorescence protein was more notably [than "S65T variant" which emitted bright fluorescence and this fluorescence moreover used as contrast also within an animal cell] strong.

[0010] Namely, this invention serves as a fluorescence mold under an elevated temperature (37 degrees C), and can emit strong fluorescence. Also in an animal cell, it is related with applicable fluorescence protein and its directions. And more specifically (1) Fluorescence protein which includes an amino acid sequence of a publication in the array number 2, (2) In an amino acid sequence given in the array number 2 1 or some amino acid Deletion, Fluorescence protein including an amino acid sequence (however, the 65th place is a threonine and the 147th place is a proline) replaced or added, (3) DNA which carries out the code of the fluorescence protein of a publication to (1) or (2), Vector which contains DNA of a publication in (4) and (3) (5) It is characterized by having arranged DNA given in (3) on a **-ed promotor's lower stream of a river. (4) Vector of a publication (6) A host cell which holds a vector of a publication to (4), (7) Include a process which cultivates a host cell of a publication to (6) and collects produced protein. (1) Or a manufacture method of fluorescence protein given in (2) (8) A vector given in (5) is introduced into a host cell. A measuring method of the activity of a **-ed promotor including a process in which fluorescence emitted from this cell is detected, (9) (1) characterized by uniting with a **-ed amino acid sequence, or fluorescence protein given in (2), (10) Fluorescence protein given in (9) is introduced into a cell, and it is characterized by observing distribution in this intracellular one of this fluorescence protein. How to detect targetting activity in intracellular [of a **-ed amino acid sequence], (11) A vector in which DNA which carries out the code of the fluorescence protein of a publication to (9) was inserted possible [a manifestation] is introduced into a host cell. It is related with a method of detecting targetting activity in intracellular [of a **-ed amino acid sequence] characterized by observing distribution in this intracellular one of this fluorescence protein.

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[0012] Therefore, in the protein of this invention, the 65th place of an amino acid sequence is a threonine, it is important that the 147th place is a proline, and the same protein is also contained in the range of this invention as intrinsically as the 65th place and the protein of this invention suitably obtained substitute, deletion, and by adding in the amino acid of an except the 147th place. As for "being the same protein intrinsically", the amino acid which does not influence

fluorescence substantially points out substitute, deletion, or the added protein among the amino acid in the amino acid sequence of the protein of this invention here.
[0013] In addition, it is possible to build DNA which carries out the code of such alteration protein easily using the site-directed-mutagenesis method which is common knowledge technology (experimental-medicine separate volume gene engineering handbook 246-258 (1991)), if it is this contractor, and to obtain the protein of this invention.
[0014] The protein of this invention can be manufactured by the following methods. First, GFPcDNA which replaced the base sequence corresponding to the 65th serine and the 147th serine by the base sequence corresponding to a threonine and a proline among the base sequences (array number: 1) of a wild type GFPcDNA, respectively is produced. Production of this GFPcDNA can be carried out, when the 193rd "the T" of a wild type GFP ("A" of a translation initiation codon "ATG" is set to 1) is replaced by "A" and it replaces the 439th "the T" by "C" by the site-directed-mutagenesis introducing method etc. Subsequently, produced cDNA is included in a suitable expression vector, it introduces into a host cell, and the protein of this invention is made to discover by host intracellular.
[0015] As an expression vector, although there is especially no limit, it sets to Escherichia coli. For example, "pQE30", "pQE31", "pQE32" (QIAGEN K.K. make), Vectors, such as "pET3a", "pET3b", "pET3c" (novagen company make, TAKARA SHUZO), "pGEX-5X-1", "pGEX-5X-2", "pGEX-5X-3" (Pharmacia manufacture), and "pUC118" (TAKARA SHUZO), are used suitably.
[0016] Installation to the host cell of a vector can be performed by methods, such as a conventional method, for example, a calcium phosphate method, the electroporation method, etc.
[0017] Moreover, a vector is introduced, there is especially no limit as a host cell for making the protein of this invention discover, and "HB [101]", "DH5", "TG1", "JM109", "XL1-blue", "BL21 (DE3)", "BL21(DE3) pLysS", etc. can be used in Escherichia coli, for example.
[0018] The protein of this invention made to discover by host intracellular is refined by the following methods, and can be collected. That is, if the protein of this invention exists as fusibility protein by host intracellular, after destroying a host cell by ultrasonic crushing etc., it is possible to refine by methods, such as ammonium sulfate precipitation and a DEAE cellulose column chromatography. Moreover, if it exists as insoluble protein, it is possible to refine [by dialysis] salt concentration for example, using the same method as the above about the protein by which lowering solubilization is carried out gradually, after solubilizing using modifiers, such as a guanidine hydrochloric acid. In addition, when the protein of this invention is fusion protein with "His-tag" (QIAGEN K.K. make), it is possible to refine with the affinity column chromatography which used "nickel-NTA resin" (QIAGEN K.K. make). Moreover, when it is fusion protein with a glutathione S-transferase (GST), it is possible to perform purification using the affinity which used the glutathione sepharose column.
[0019] Especially the protein of this invention has the high utility value as an indicator. That is, if the protein of this invention is refined as fusion protein with a **-ed amino acid sequence, it introduces into intracellular by technique, such as a microinjection method, and distribution of this fusion protein is observed with time, it is possible to detect the targetting activity in intracellular [of a **-ed amino acid sequence]. As a **-ed amino acid sequence, although there is especially no limit, a targeting signal (for example, a nuclear shift signal, a mitochondrion pre array) etc. is suitable, for example. In addition, besides introducing into intracellular by a microinjection method etc., it is made discovered by intracellular and the protein of this invention can also be used. In this case, the vector in which DNA which carries out the code of the protein of this invention was inserted possible [a manifestation] is introduced into a host cell.
[0020] Moreover, the protein of this invention can also be used for measurement of promotor activity as reporter protein. That is, it is possible to measure a **-ed promotor's activity by building the vector by which DNA which carries out the code of the protein of this invention to a **-ed promotor's lower stream of a river has been arranged, introducing this into a host cell, and detecting the fluorescence of the protein of this invention emitted from this cell. As a **-ed promotor, if it functions by host intracellular, there will be especially no limit.
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[0025] In addition, DNA which carries out the code of the protein of this invention can also be used for the analysis of the molecule-molecule interaction using the analysis and the energy transfer of folding (folding) of protein.

[0026]

[Example] Hereafter, although an example explains this invention concretely, this invention is not limited to the following examples.

[0027] [example 1] In order to amplify GFPcDNA into which a random variation was introduced, GFPcDNA of a wild type is used as mold. cloning of cDNA which carries out the code of the GFP with high fluorescence intensity -- 5' sense primer (a 4:5'-GGGCCCGGATCCATGAGTAAAGGAGAAGAACTTTTC-3':7-12 position base is BamHI site (underline portion), and the 13-15th is an initiation codon. array number:) And a 3' antisense primer (5:5'-GCGCACGGTACCTTATTTGTATAGTTCATCCATGCCATG-3':7-12 position is KpnI site (underline portion) array number:) the 13-15th is a termination codon. It used and "Low fidelity PCR amplification" was performed. Being concrete and the reaction solution of 100microl (Tris-Cl (pH8:3) each dNTP of the template DNA "pAGN1" of 100ng, each oligonucleotide primer of 1mM, and 1mM and 10mM) As opposed to MgCl2 of KCl of 50mM, and 1.5mM, MnCl2 of 0.5mM, and 1ml "TaKaRa Taq" (TAKARA SHUZO) It is 30 cycle ****** about processing of "being [degrees C / 94] 3 minutes (it being 10 minutes at 72 ** in the last cycle) at 2 minutes and 72 ** in 1 minute and 55 degree C." "Ligation high" (Toyobo) was used for the expression vector "pQE30" (product made from Qiagen) of Escherichia coli, and the PCR product acquired by this was inserted in it.

[0028] Subsequently, the obtained plasmid was introduced into the competent cell of Escherichia coli XL1-blue, and it scattered 300 colonies at a time on ten plates. UV irradiation was performed after culture of 20 hours at 37 degrees C, and the colony which showed bright fluorescence especially was isolated. The base sequence was determined using "DNA Sequencing Kit 402079" (PerkinElmer, Inc. make), having used synthetic oligo as the probe for the plasmid which collected plasmids and was obtained from the isolated colony.

[0029] Consequently, the clone (this clone is hereafter called "S147P variant") which mutated the 147th serine of GFP of a wild type to the proline was obtained.

[0030] [Example 2] It had variation into portions other than the array of the chromophore considered that preparation of GFP which has two variation, S147P variation and S65T variation, and measurement "S147P variant" of this fluorescence intensity of GFP have determined the fluorescence of GFP. Then, this invention person produced cDNA of GFP (this variant is hereafter called "S65 T/S147P variant") which has "S65T variation" of "S65T variant" and two variation of the above "S147P variation" which are widely put in practical use in the variant of the GFP chromophore array which emits strong fluorescence.

[0031] The plasmid which included the variant GFP which changed the 65th serine of a wild type GFP into the threonine (S65T) in the BamHI part of "pRSETB" (in vitro gene company make) "pRSETB-GFP (S65T)" (Nature 373 663-664 (1995): a grant was made by Dr. Roger Y.Tsien of University of California.) It considers as mold. in addition, the site-specific mutation introducing method of the above [this plasmid] based on "pGFPcDNA vector" (Clontech make) -- it can prepare -- The primer which includes a SmaI site in this 5' side of GFP (a 6:5'-TTCACCCGGGATGAGTAAAGGAGAAGAACTT-3':5-10 position base is SmaI site (underline portion) array number:) The 11-13th is an initiation codon. The primer which includes an EcoRI site in 3' side (a 7:5'-GCACGAATTCTATTTGTATAGTTCATCCATGCC-3':5-10 position base is EcoRI site (underline portion) array number:) the 19-21st -- a termination codon -- it is -- after producing and performing PCR, it digested by SmaI and EcoRI and "pGEM-GFP (S65T)" which inserted the fragment in SmaI of "pGEM-7Zf(+)" (pro megger company make) and an EcoRI site was produced. "pGEM-GFP (S65T)" was digested by SmaI and XbaI, cloning of the GFP fragment was carried out to SmaI of "pUC118" (TAKARA SHUZO CO., LTD. make), and a XbaI site, and "pUCGFP (S65T)" was produced. Furthermore, "pUCGFP (S65T)" was digested by KpnI and HindIII, cloning of the GFP fragment was carried out to KpnI of "pQE31" (QIAGEN K.K.), and a HindIII site, and "pQE31GFP (S65T)" was produced. The plasmid which connected the small fragment which carried out NdeI digestion of the large fragment after digestion and by the side of the frame and the "pQA2 (pQE30GFP (S147P))" for "pQE31GFP (S65T)" by NdeI using the ligase, and "pQB2 (pQE31GFP (S65 T/S147P))" with two variation were built.
[0032] Subsequently, the obtained plasmid was introduced into the competent cell of Escherichia coli XL1-blue, 100micro (LB culture medium) of biomasses 1 cultivated at 37 degrees C all night was planted in 10ml LB culture medium, and 180rpm performed shaking culture at next 37 degrees C for 3 hours. subsequently, a cell -- gathering a harvest -- the buffer A of 100microl (Tris-Cl (pH7.5) of 50mM, 20mM EDTA, 2mM PMSF) -- final -- "-- it suspended so that it might become OD600=about 0.5", and SDS (0.1% of final concentration) was added, centrifugal [of this cell suspension] was carried out by 15,000G after the vortex for 10 minutes, and that supernatant liquid was used for fluorescence intensity measurement and GFP density measurement.
[0033] In addition, fluorescence intensity measurement was performed on the excitation wavelength of 480nm, and the fluorescence wavelength of 510nm using "DensitographLumino-CCD" (Atto). Moreover, GFP concentration was measured using the ECL Western-blotting detection kit (Amersham make) which used chemiluminescence as a secondary antibody, using a rabbit anti-GFP antibody (Clonetec make) as a primary antibody. The above result is shown in a table 1. In addition, all the units of the numeric character in a table used the relative value.
[0034]
[A table 1]

GFP	蛍光強度	GFP蛋白濃度	蛍光強度／GFP蛋白濃度
S65T	244	40	6.10
S65T/S147P	1321	73	18.09
GFPなし	11	3	

Both 37-degree C GFP concentration (intracellular abundance) and fluorescence intensity went up notably by installation of S147P variation so that clearly from a table 1. Moreover, the fluorescence intensity per GFP concentration also increased by about 3 times as compared with S65T conventional variant.
[0035] [Example 3] cDNA of measurement "S65 T/S147P variant" of the excitation and the

fluorescence spectrum of "S65 T/S147P variant" and an "S65T variant" was included in "pGEX5X-2" (Pharmacia manufacture), it introduced into Escherichia coli, and "S65 T/S147P variant" was made to discover as fusion protein with GST (glutation S-transferase). The glutathione sepharose 4B column (Pharmacia manufacture) refined this, and excitation and the fluorescence spectrum of fusion protein were measured. In addition, "S65T variant" was used as contrast. Consequently, about excitation wavelength, "S65 T/S147P" was not accepted, and the big difference was not accepted for "S65T" among both by 490nm. [496nm] Moreover, both of the peak of fluorescence wavelength were the same as that of 512nm (drawing 1). It became clear that it is possible to excite "S65 T/S147P" by the completely same method as "S65T" from this, and to measure fluorescence. In addition, in all spectrums, the axis of ordinate set the peak to 1.0.

[0036] the animal cell expression vector "pUC-CAGGS(Xhol)" (it Miura(s) "pCAGGS":I.Miyamoto and N. --) digested by the effect EcoRI in the mammalian cell of [example 4] "S65 T/S147P variant" H. Niwa, J.Miyazaki, and and KTanaka (1992) J.Biol.Chem.267 : cDNA of the "S65 T/S147P variant" which used "Ligation high" (Toyobo) for 12182-12187; and was digested by EcoRI is inserted. Passing away-transfection was carried out with calcium settling at L cell of the mouse origin. After cultivating the cell at 37 degrees for 48 hours, it fixed with formalin 10%, and the fluorescence microscope detected the NOMARU skiing (Nomarski) image and the fluorescence image (fluorescence of GFP) in an FITC filter (drawing 2 A lower berth). In addition, cDNA of "S65T variant" was used as contrast (drawing 2 A upper case). Consequently, as compared with "S65T variant", the cell which discovers "S65 T/S147P variant" showed the brighter fluorescence image (drawing 2 A lower right).

[0037] Moreover, the strength of the rate of the cell which emits fluorescence among the observed cells, and the fluorescence of a cell was measured (drawing 2 B). "S65 T/S147P" shows fluorescence intensity and, as for "S65 T/S147P" when the horizontal axis of drawing sets to 1 fluorescence intensity of the cell which was the strongest as for fluorescence, the axis of ordinate of drawing shows the number of cells of a fluorescence cell.

[0038] Consequently, in the cell in which cDNA of "S65 T/S147P variant" was inserted, the cell had emitted fluorescence at a higher rate as compared with contrast. Moreover, fluorescence intensity was also notably high as compared with contrast.

[0039]

[Effect of the Invention] The protein in which the 65th and the 147th amino acid of a wild type GFP were replaced by the threonine and the proline by this invention, respectively was offered. the advanced type which the protein of this invention became a fluorescence mold under the 37-degree C temperature condition, and has been used widely conventionally -- since the amount of manifestations as fusibility protein was also increasing about 2 times while emitting about 3 times as much strong fluorescence as GFP, it became clear to emit fluorescence bright about about 6 times at 37 degrees C as a result compared with the conventional type. Compared with the conventional thing, it is thought from the ability of not only a microorganism but an animal cell to be applied [that a 37-degree C difference is remarkable and] that this advanced GFP is effective in the yeast which can be especially grown at an animal cell or a broad temperature. It uses GFP of this invention as a proteinic indicator, and it is not only suitable for the object which observes the localization of the molecule in a viable cell, but it is thought that it is effective also as the reporter protein in promotor analysis and a marker of higher-order-structure change of protein, and the utilization in cell biology and the gene engineering field will be expected widely from now on.

[0040]

[Layout Table]

array number: -- CDS existence location: -- 1.. 714 feature : Length of 1 array : Mold of 717 arrays : The number of nucleic-acid chains : Double strand topology : Class of straight chain-like array : Mark showing the feature feature of a cDNA to mRNA array Determined method: E ** Train ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT 48 Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro IleLeu Val 1 5 10 15 GAA TTA GAT

GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG 96 Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu 20 25 30 GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC 144 Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr LeuLys PheIle Cys 35 40 45 ACT ACT GGA AAA CTA CCTGTT CCA TGG CCA ACA CTT GTC ACT ACT TTC 192 Thr Thr Gly Lys Leu Pro Val Pro TrpPro Thr Leu Val Thr Thr Phe 50 55 60 TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG 240 Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg 65 70 75 80 CATGAC TTT TTC AAG AGT GCC ATG CCC GAA GGTTAT GTA CAG GAA AGA 288 His Asp Phe Phe Lys Ser Ala Met ProGlu Gly Tyr Val Gln Glu Arg 85 90 95 ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGTGCT GAA GTC 336 Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr ArgAla Glu Val 100 105 110 AAG TTTGAA GGT GAT ACC CTT GTT AATAGA ATC GAG TTA AAA GGT ATT 384 Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile 115 120 125 GAT TTT AAA GAA GAT GGA AAC ATT CTT GGACAC AAA TTG GAA TAC AAC 432 Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn 130 135 140 TATAAC TCA CAC AAT GTATAC ATC ATG GCA GAC AAA CAA AAG AAT GGA 480 Tyr Asn Ser His Asn Val Tyr Ile MetAla Asp Lys Gln Lys Asn Gly 145 150 155 160 ATC-AAA-GTT-AAC-TTC AAA ATT AGA CAC AAC-ATT-GAA-GAT-GGA-AGC-GTT 528Ile Lys Val Asn Phe-Lys-Ile-Arg-His Asn Ile Glu Asp Gly-Ser-Val 165 170 175 CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT 576 Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro 180 185 190 GTCCTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG 624 Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser 195 200 205 AAAGAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA 672 Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val 210 215 220 ACAGCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA 714 Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 225 230 235 TAA 717 array number : Length of 2 arrays : Mold of 238 array : Amino acid topology : Class of straight chain-like array : Protein ** Train Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val 1 5 10 15 Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe SerVal Ser Gly Glu 20 25 30 Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys 35 4045 Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe 5055 60 Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg 65 70 75 80 His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg 85 90 95 Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val 100 105 110 Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile 115 120 125 Asp-Phe-Lys-Glu-Asp Gly Asn Ile Leu Gly-His-Lys-Leu-Glu-Tyr-Asn 130 135 140 Tyr-Asn-Pro-His-Asn Val Tyr Ile Met Ala-Asp-Lys-Gln-Lys-Asn-Gly 145 150155 160 Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val 165170 175 Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro 180 185 190 ValLeu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser 195 200 205 Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val 210 215 220 Thr AlaAla Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 225 230 235 array number CDS existence location : 1.. 714 feature : The length of 3 arrays : Mold of 717 arrays : The number of nucleic-acid chains : Double strand topology : Class of straight chain-like array : Mark showing the feature feature of a cDNA to mRNA array : Determined method: E ** Train ATG AGT AAA GGA GAA GAACTT TTC ACT GGA GTT GTC CCA ATT CTT GTT 48 Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val 1 5 10 15 GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG 96 Glu Leu Asp Gly Asp Val Asn GlyHis Lys Phe Ser Val Ser Gly Glu 20 25 30 GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC 144 Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr LeuLys PheIle Cys 35 40 45 ACT ACT GGA AAACTA CCTGTT CCA TGG CCA ACA CTT GTC ACT ACT TTC 192 Thr Thr Gly Lys Leu Pro Val ProTrp Pro Thr Leu Val Thr Thr Phe 50 55 60 ACT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG 240 Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg 65 70 75 80 CAT-GAC-TTT-TTC-AAG AGT GCC ATG CCC GAA-GGT-TAT-GTA-CAG-GAA-AGA 288His Asp Phe Phe Lys-Ser-Ala-Met-Pro Glu Gly Tyr Val Gln-Glu-Arg 85 90 95 ACT ATA

TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC 336 Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val100 105 110 AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTAAAA GGT ATT 384 Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile 115 120 125 GAT TTT AAAGAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC 432 Asp Phe Lys Glu Asp Gly Asn Ile Leu GlyHis Lys Leu Glu Tyr Asn 130 135 140 TATAAC CCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA 480 Tyr Asn Pro His Asn Val Tyr Ile MetAla Asp Lys Gln Lys Asn Gly 145 150 155 160 ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT 528 Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val 165 170 175 CAACTA GCA GAC CAT TAT CAA CAA AAT ACTCCA ATT GGC GAT GGC CCT 576 Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro 180 185 190 GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACACAA TCT GCC CTT TCG 624 Val Leu Leu Pro Asp Asn His Tyr Leu SerThr Gln Ser Ala Leu Ser 195 200 205 AAAGAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA 672 Lys Asp Pro Asn Glu Lys Arg Asp His Met ValLeu Leu Glu Phe Val 210 215 220 ACAGCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA 714 Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys 225 230 235 TAA717 array number : The length of 4 arrays : Mold of 36 arrays : The number of nucleic-acid chains : Single strand topology : Class of straight chain-like array : others -- nucleic acid ****DNA** Train GGGCCCGGAT CCATGAGTAA AGGAGAAGAA CTTTTC 36 array number :. Length of 5 arrays : Mold of 39 arrays : The number of nucleic-acid chains : Single strand topology : Class of straight chain-like array : others -- nucleic acid Synthetic DNA ** Train GCGCACGGTA CCTTATTTGT ATAGTTCATC CATGCCATG 39 array number : Length of 6 arrays : Mold of 31 arrays : The number of nucleic-acid chains : Single strand topology : Class of straight chain-like array : others -- nucleic acid ****DNA** Train TTCACCCGGG ATGAGTAAAG GAGAAGAACT T 31 array number: Length of 7 arrays : Mold of 33 arrays : The number of nucleic-acid chains : Single strand topology : Class of straight chain-like array : others -- nucleic acid ****DNA** Train GCACGAATTC TATTTGTATA GTTCATCCAT GCC 33

[Translation done.]

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EXAMPLE

[Example] Hereafter, although an example explains this invention concretely, this invention is not limited to the following examples.
[0027] [example 1] In order to amplify GFPcDNA into which a random variation was introduced, GFPcDNA of a wild type is used as mold. cloning of cDNA which carries out the code of the GFP with high fluorescence intensity -- 5' sense primer (a 4:5'-GGGCCCGGATCCATGAGTAAAGGAGAAGAACTTTTC-3':7-12 position base is BamHI site (underline portion), and the 13-15th is an initiation codon. array number:) And a 3' antisense primer (5:5'-GCGCACGGTACCTTATTTGTATAGTTCATCCATGCCATG-3':7-12 position is KpnI site (underline portion) array number:) the 13-15th is a termination codon. It used and "Low fidelity PCR amplification" was performed. Being concrete and the reaction solution of 100microl (Tris-Cl (pH8.3) each dNTP of the template DNA "pAGN1" of 100ng, each oligonucleotide primer of 1mM, and 1mM and 10mM) As opposed to MgCl2 of KCl of 50mM, and 1.5mM, MnCl2 of 0.5mM, and 1ml "TaKaRa Taq" (TAKARA SHUZO) It is 30 cycle ****** about processing of "being [degrees C / 94] 3 minutes (it being 10 minutes at 72 ** in the last cycle) at 2 minutes and 72 ** in 1 minute and 55 degree C." "Ligation high" (Toyobo) was used for the expression vector "pQE30" (product made from Qiagen) of Escherichia coli, and the PCR product acquired by this was inserted in it.
[0028] Subsequently, the obtained plasmid was introduced into the competent cell of Escherichia coli XL1-blue, and it scattered 300 colonies at a time on ten plates. UV irradiation was performed after culture of 20 hours at 37 degrees C, and the colony which showed bright fluorescence especially was isolated. The base sequence was determined using "DNA Sequencing Kit 402079" (PerkinElmer, Inc. make), having used synthetic oligo as the probe for the plasmid which collected plasmids and was obtained from the isolated colony.
[0029] Consequently, the clone (this clone is hereafter called "S147P variant") which mutated the 147th serine of GFP of a wild type to the proline was obtained.
[0030] [Example 2] It had variation into portions other than the array of the chromophore considered that preparation of GFP which has two variation, S147P variation and S65T variation, and measurement "S147P variant" of this fluorescence intensity of GFP have determined the fluorescence of GFP. Then, this invention person produced cDNA of GFP (this variant is hereafter called "S65 T/S147P variant") which has "S65T variation" of "S65T variant" and two variation of the above "S147P variation" which are widely put in practical use in the variant of the GFP chromophore array which emits strong fluorescence.
[0031] The plasmid which included the variant GFP which changed the 65th serine of a wild type GFP into the threonine (S65T) in the BamHI part of "pRSETB" (in vitro gene company make) "pRSETB-GFP (S65T)" (Nature 373 663-664 (1995): a grant was made by Dr. Roger Y.Tsien of University of California.) It considers as mold. in addition, the site-specific mutation introducing method of the above [this plasmid] based on "pGFPcDNA vector" (Clontech make) -- it can prepare -- The primer which includes a SmaI site in this 5' side of GFP (a 6:5'-TTCACCCGGGATGAGTAAAGGAGAAGAACTT-3':5-10 position base is SmaI site (underline portion) array number:) The 11-13th is an initiation codon. The primer which includes an EcoRI site in 3' side (a 7:5'-GCACGAATTCTATTTGTATAGTTCATCCATGCC-3':5-10 position base is EcoRI site (underline portion) array number:) the 19-21st -- a termination codon -- it is -- after

producing and performing PCR, it digested by SmaI and EcoRI and "pGEM-GFP (S65T)" which inserted the fragment in SmaI of "pGEM-7Zf(+)" (pro megger company make) and an EcoRI site was produced. "pGEM-GFP (S65T)" was digested by SmaI and XbaI, cloning of the GFP fragment was carried out to SmaI of "pUC118" (TAKARA SHUZO CO., LTD. make), and a XbaI site, and "pUCGFP (S65T)" was produced. Furthermore, "pUCGFP (S65T)" was digested by KpnI and HindIII, cloning of the GFP fragment was carried out to KpnI of "pQE31" (QIAGEN K.K.), and a HindIII site, and "pQE31GFP (S65T)" was produced. The plasmid which connected the small fragment which carried out NdeI digestion of the large fragment after digestion and by the side of the frame and the "pQA2 (pQE30GFP (S147P))" for "pQE31GFP (S65T)" by NdeI using the ligase, and "pQB2 (pQE31GFP (S65 T/S147P))" with two variation were built.

[0032] Subsequently, the obtained plasmid was introduced into the competent cell of Escherichia coli XL1-blue, 100micro (LB culture medium) of biomasses 1 cultivated at 37 degrees C all night was planted in 10ml LB culture medium, and 180rpm performed shaking culture at next 37 degrees C for 3 hours. subsequently, a cell -- gathering a harvest -- the buffer A of 100microl (Tris-Cl (pH7.5) of 50mM, 20mM EDTA, 2mM PMSF) -- final -- "-- it suspended so that it might become OD600=about 0.5", and SDS (0.1% of final concentration) was added, centrifugal [of this cell suspension] was carried out by 15,000G after the vortex for 10 minutes, and that supernatant liquid was used for fluorescence intensity measurement and GFP density measurement.

[0033] In addition, fluorescence intensity measurement was performed on the excitation wavelength of 480nm, and the fluorescence wavelength of 510nm using "DensitographLumino-CCD" (Atto). Moreover, GFP concentration was measured using the ECL Western-blotting detection kit (Amersham make) which used chemiluminescence as a secondary antibody, using a rabbit anti-GFP antibody (Clonetec make) as a primary antibody. The above result is shown in a table 1. In addition, all the units of the numeric character in a table used the relative value.

[0034]

[A table 1]

GFP	蛍光強度	GFP蛋白濃度	蛍光強度／GFP蛋白濃度
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Both 37-degree C GFP concentration (intracellular abundance) and fluorescence intensity went up notably by installation of S147P variation so that clearly from a table 1. Moreover, the fluorescence intensity per GFP concentration also increased by about 3 times as compared with S65T conventional variant.

[0035] [Example 3] cDNA of measurement "S65 T/S147P variant" of the excitation and the fluorescence spectrum of "S65 T/S147P variant" and an "S65T variant" was included in "pGEX5X-2" (Pharmacia manufacture), it introduced into Escherichia coli, and "S65 T/S147P variant" was made to discover as fusion protein with GST (glutation S-transferase). The glutathione sepharose 4B column (Pharmacia manufacture) refined this, and excitation and the fluorescence spectrum of fusion protein were measured. In addition, "S65T variant" was used as contrast. Consequently, about excitation wavelength, "S65 T/S147P" was not accepted, and the big difference was not accepted for "S65T" among both by 490nm. [496nm] Moreover, both of the peak of fluorescence wavelength were the same as that of 512nm (drawing 1). It became clear that it is possible to excite "S65 T/S147P" by the completely same method as "S65T" from this, and to measure fluorescence. In addition, in all spectrums, the axis of ordinate set the peak to

1.0.
[0036] the animal cell expression vector "pUC-CAGGS(XhoI)" (it Miura(s) "pCAGGS":I.Miyamoto and N. --) digested by the effect EcoRI in the mammalian cell of [example 4] "S65 T/S147P variant" H. Niwa, J.Miyazaki, and and KTanaka (1992) J.Biol.Chem.267 : cDNA of the "S65 T/S147P variant" which used "Ligation high" (Toyobo) for 12182-12187, and was digested by EcoRI is inserted. Passing away-transfection was carried out with calcium settling at L cell of the mouse origin. After cultivating the cell at 37 degrees for 48 hours, it fixed with formalin 10%, and the fluorescence microscope detected the NOMARU skiing (Nomarski) image and the fluorescence image (fluorescence of GFP) in an FITC filter (drawing 2 A lower berth). In addition, cDNA of "S65T variant" was used as contrast (drawing 2 A upper case). Consequently, as compared with "S65T variant", the cell which discovers "S65 T/S147P variant" showed the brighter fluorescence image (drawing 2 A lower right).
[0037] Moreover, the strength of the rate of the cell which emits fluorescence among the observed cells, and the fluorescence of a cell was measured (drawing 2 B). "S65 T/S147P" shows fluorescence intensity and, as for "S65 T/S147P" when the horizontal axis of drawing sets to 1 fluorescence intensity of the cell which was the strongest as for fluorescence, the axis of ordinate of drawing shows the number of cells of a fluorescence cell.
[0038] Consequently, in the cell in which cDNA of "S65 T/S147P variant" was inserted, the cell had emitted fluorescence at a higher rate as compared with contrast. Moreover, fluorescence intensity was also notably high as compared with contrast.

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CLAIMS

[Claim(s)]
[Claim 1] Fluorescence protein which includes an amino acid sequence of a publication in an array number 2.
[Claim 2] Fluorescence protein with which 1 or some amino acid include deletion and an amino acid sequence (however, the 65th place is a threonine and the 147th place is a proline) replaced or added in an array number 2 in an amino acid sequence of a publication.
[Claim 3] DNA which carries out the code of the fluorescence protein according to claim 1 or 2.
[Claim 4] A vector containing DNA according to claim 3.
[Claim 5] A vector according to claim 4 characterized by having arranged DNA according to claim 3 on a **-ed promotor's lower stream of a river.
[Claim 6] A host cell holding a vector according to claim 4.
[Claim 7] A manufacture method of fluorescence protein including a production process which cultivates a host cell according to claim 6, and collects produced protein according to claim 1 or 2.

[Claim 8] A measuring method of the activity of a **-ed promotor who introduces a vector according to claim 5 into a host cell, and includes a process in which fluorescence emitted from this cell is detected.
[Claim 9] Fluorescence protein according to claim 1 or 2 characterized by uniting with a **-ed amino acid sequence.
[Claim 10] How to detect targetting activity in intracellular [of a **-ed amino acid sequence] which introduces fluorescence protein according to claim 9 into a cell, and is characterized by observing distribution in this intracellular one of this fluorescence protein.
[Claim 11] How to detect targetting activity in intracellular [of a **-ed amino acid sequence] which introduces into a host cell a vector in which DNA which carries out the code of the fluorescence protein according to claim 9 was inserted possible [a manifestation], and is characterized by observing distribution in this intracellular one of this fluorescence protein.

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is drawing showing the measurement result of the excitation and the fluorescence spectrum of "S65 T/S147P variant" and an "S65T variant."
[Drawing 2] Drawing 2 A is a microphotography in which a fluorescence microscope detects the cell into which cDNA of "S65 T/S147P variant" and an "S65T variant" was introduced, and the NOMARU skiing image and a fluorescence image are shown. Drawing 2 B is drawing of the cell which emits fluorescence in a tested cell in which reaching comparatively and showing the measurement result of the strength of the fluorescence of the cell.

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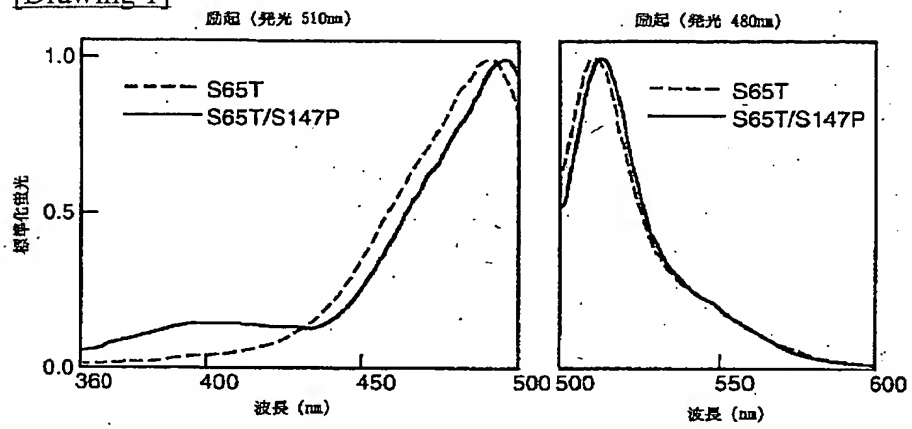
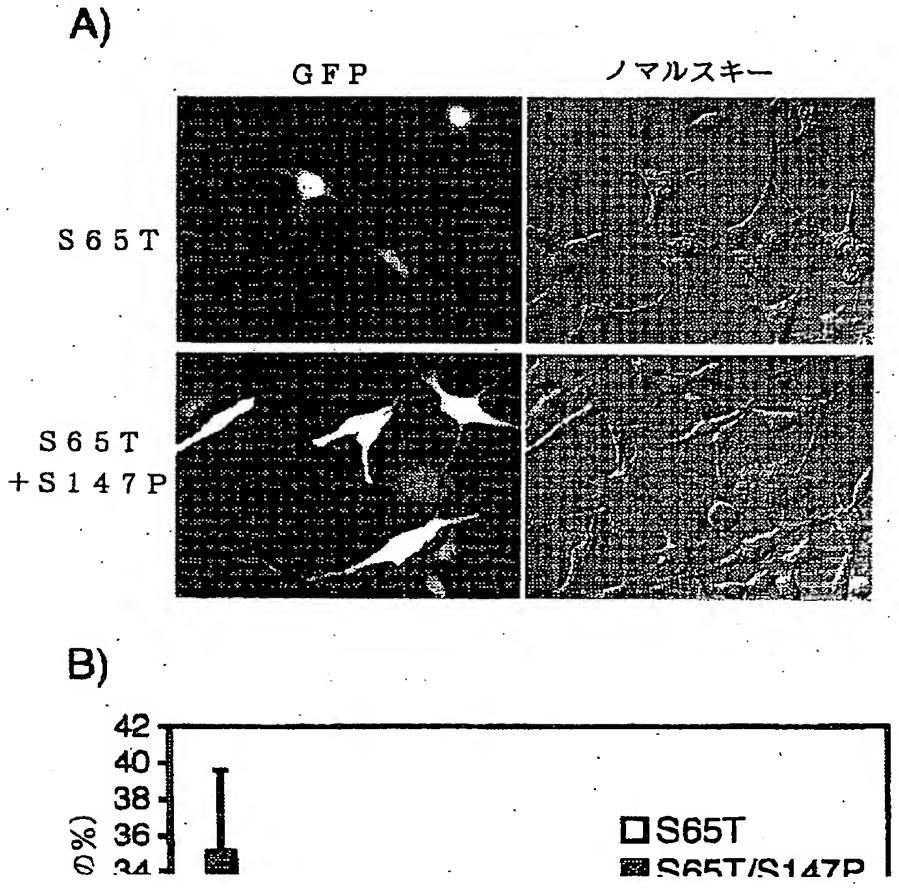
DRAWINGS

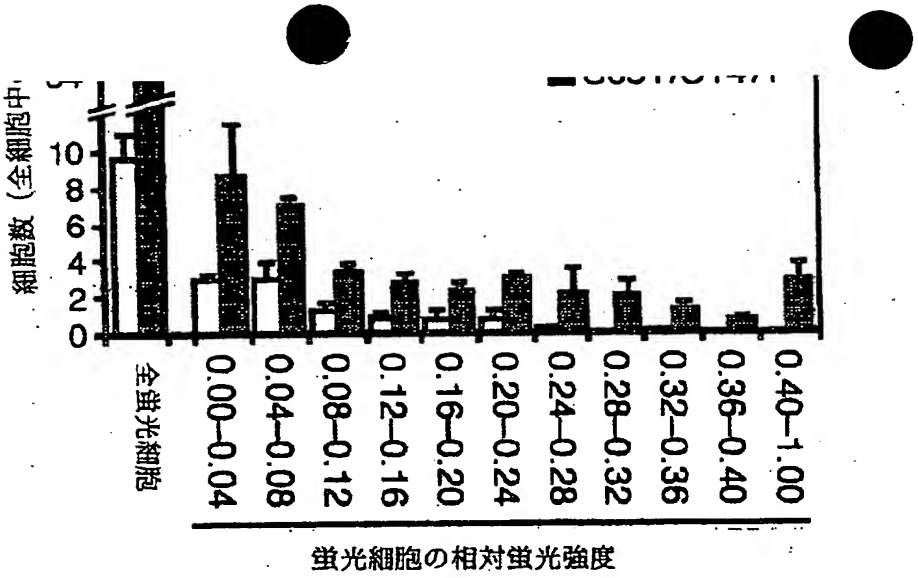
[Drawing 1]



[Drawing 2]

図面代用写真





[Translation done.]

ITで消化した「S65T/S147P変異体」のcDNAを挿入し、マウス由来のL cellにカルシウム沈殿法で一過的トランスフェクトした。その細胞を37度で48時間培養した後に10%ホルマリンで固定し、蛍光顕微鏡によりノマルスキー(Nomarski)像およびFITCフィルターでの蛍光像(GFPの蛍光)を検出した（図２Ａ下段）。なお、対照として「S65T変異体」のcDNAを用いた（図２Ａ上段）。この結果、「S65T変異体」と比較して、「S65T/S147P変異体」を発現する細胞は、より明るい蛍光像を示した（図２Ａ右下）。

【００３７】また、観察した細胞の内、蛍光を発する細胞の割合、及び細胞の蛍光の強さを測定した（図２Ｂ）。図の横軸は、最も蛍光の強かった細胞の蛍光強度を１とした場合における「S65T/S147P」は「S65T/S147P」は蛍光強度を示し、図の縦軸は、蛍光細胞の細胞数を示す。

【００３８】この結果、「S65T/S147P変異体」のcDNAを挿入された細胞では、対照と比較して、より高い割合で細胞が蛍光を発していた。また、蛍光強度も対照と比較して顕著に高かった。

【００３９】

【発明の効果】本発明により野生型GFPの65番目と147番目のアミノ酸がそれぞれトレオニン、プロリンに置換されたタンパク質が提供された。本発明のタンパク質は、37℃の温度条件下においても蛍光型となり、また従来広く用いられてきた改良型ＧＦＰの約3倍の強い蛍光を発＊すると共に可溶性タンパク質としての発現量も２倍程度増加しているため、従来のタイプに比べ結果として37℃で約6倍程度明るい蛍光を発することが明らかとなった。この改良型GFPは従来のものに比べ37℃での差が顕著であること、微生物のみならず動物細胞でも適用可能であることから、特に動物細胞や幅広い温度で生育可能な酵母などに有効と考えられる。本発明のGFPは、タンパク質の標識として用い、生細胞における分子の局在を観察する目的に適しているだけでなく、プロモーター解析におけるレポータータンパク質として、またタンパク質の高次構造変化のマーカーとしても有効と考えられ、今後広く細胞生物学、遺伝子工学分野においての利用が期待される。

【００４０】

【配列表】

配列番号： 1
配列の長さ： 717
配列の型 ：核酸
鎖の数 ：二本鎖
トポロジー ：直鎖状
配列の種類 ： cDNA to mRNA
配列の特徴
特徴を表す記号： CDS
存在位置： 1..714
特徴を決定した方法： E

配 列

```
ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT      48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
1               5                  10                  15
GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG      96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30
GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC     144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45
ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC     192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60
TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG     240
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
65                  70                  75                  80
CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA     288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95
ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC     336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110
AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT     384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
```

インフォメーション

☞ 入力データに誤りがあります。

13 14

180 185 190
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
195 200 205
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
210 215 220
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225 230 235

配列番号： 3
配列の長さ： 717
配列の型：核酸
鎖の数：二本鎖
トポロジー：直鎖状

＊配列の種類： cDNA to mRNA
配列の特徴
10 特徴を表す記号： CDS
存在位置： 1..714
＊ 特徴を決定した方法： E

配 列

```
ATG AGT AAA GGA GAA GAA CTT TTC ACT GGA GTT GTC CCA ATT CTT GTT      48
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
1               5                  10                  15
GAA TTA GAT GGT GAT GTT AAT GGG CAC AAA TTT TCT GTC AGT GGA GAG      96
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
            20                  25                  30
GGT GAA GGT GAT GCA ACA TAC GGA AAA CTT ACC CTT AAA TTT ATT TGC     144
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
        35                  40                  45
ACT ACT GGA AAA CTA CCT GTT CCA TGG CCA ACA CTT GTC ACT ACT TTC     192
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
    50                  55                  60
ACT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG     240
Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Arg
65                  70                  75                  80
CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA     288
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
                85                  90                  95
ACT ATA TTT TTC AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC     336
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
            100                 105                 110
AAG TTT GAA GGT GAT ACC CTT GTT AAT AGA ATC GAG TTA AAA GGT ATT     384
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
        115                 120                 125
GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA TTG GAA TAC AAC     432
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
    130                 135                 140
TAT AAC CCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA     480
Tyr Asn Pro His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145                 150                 155                 160
ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT     528
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
                165                 170                 175
CAA CTA GCA GAC CAT TAT CAA CAA AAT ACT CCA ATT GGC GAT GGC CCT     576
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
            180                 185                 190
GTC CTT TTA CCA GAC AAC CAT TAC CTG TCC ACA CAA TCT GCC CTT TCG     624
```

15 16

Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
195 200 205

AAA GAT CCC AAC GAA AAG AGA GAC CAC ATG GTC CTT CTT GAG TTT GTA 672
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
210 215 220

ACA GCT GCT GGG ATT ACA CAT GGC ATG GAT GAA CTA TAC AAA 714
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225 230 235

TAA 717

配列番号： 4 10＊鎖の数 ：一本鎖
配列の長さ： 36 トポロジー ：直鎖状
配列の型 ：核酸 ＊ 配列の種類 ： 他の核酸 合成DNA
配 列
GGGCCCGGAT CCATCAGTAA AGGAGAAGAA CTTTTC 36

配列番号： 5 ※鎖の数 ：一本鎖
配列の長さ： 39 トポロジー ：直鎖状
配列の型 ：核酸 ※ 配列の種類 ： 他の核酸 合成DNA
配 列
GCGCACGGTA CCTTATTTGT ATAGTTCATC CATGCCATG 39

配列番号： 6 20★鎖の数 ：一本鎖
配列の長さ： 31 トポロジー ：直鎖状
配列の型 ：核酸 ★ 配列の種類 ： 他の核酸 合成DNA
配 列
TTCACCCGGG ATGAGTAAAG GAGAAGAACT T 31

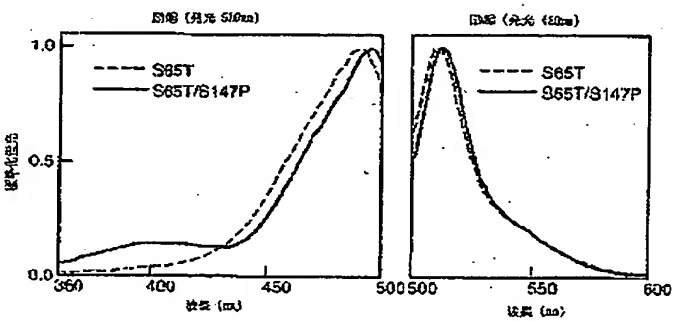
配列番号： 7 ☆鎖の数 ：一本鎖
配列の長さ： 33 トポロジー ：直鎖状
配列の型 ：核酸 ☆ 配列の種類 ： 他の核酸 合成DNA
配 列
GCACGAATTC TATTTGTATA GTTCATCCAT GCC 33

【図面の簡単な説明】

【図１】「S65T/S147P変異体」及び「S65T変異体」の励起・蛍光スペクトルの測定結果を示す図である。

【図２】図２Ａは、「S65T/S147P変異体」及び「S65T変異体」のcDNAが導入された細胞を蛍光顕微鏡により検出◆し、そのノマルスキー像及び蛍光像を示す顕微鏡写真である。図２Ｂは、被検細胞の中で蛍光を発する細胞の割合及びその細胞の蛍光の強さの測定結果を示す図である。

【図１】



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